

EXHIBIT 21

**UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF NEW YORK**

**IN RE: Acetaminophen – ASD-ADHD
Products Liability Litigation**

This Document Relates To:

All Cases

Case No. 1:22-md-03043-DLC

EXPERT REPLY REPORT OF STAN G. LOUIE, Pharm.D.

July 28, 2023

I. INTRODUCTION

A. Background

1. I have been asked by Plaintiffs' counsel in this litigation to determine, according to the publicly available evidence, the dose/duration at which prenatal (fetal) exposure to N-acetyl-p-aminophenol (APAP or acetaminophen, also known as paracetamol and by its brand name, Tylenol®) increases the risk of developing autism spectrum disorder ("ASD") and attention-deficit/hyperactivity disorder ("ADHD"). On June 16, 2023, I submitted an affirmative report on this matter disclosing my opinions. On June 21, 2023, I submitted an amended report on this matter (the "Louie Report"). I incorporate that report by reference.

2. I understand that on July 21, 2023, Defendants Johnson & Johnson Consumer, Inc. submitted the Expert Report of Mitchell R. McGill (the "McGill Report") to address the biological mechanisms following maternal ingestion of therapeutic doses of acetaminophen. I understand that Johnson & Johnson Consumer, Inc. also submitted reports from other experts, including Dr. Craig M. Powell (the "Powell Report"), which also took issue with some of my opinions set forth in the Louie Report. While I focus on the opinions of Drs. McGill and Powell in my report below, I note that there are flaws in many of Defendants' experts' reports, and the fact that I have not responded specifically to a point or criticism raised by another expert is not a concession of the point or an indication that I do not disagree with those experts' view. Although I address many of these opinions below, this reply report is not an exhaustive response to every expert offered by Defendants. I have been asked by Plaintiffs' counsel to analyze and respond to the McGill Report and portions of the Powell Report. My opinions and the bases for these opinions regarding the McGill Report and Powell Report are contained in the remainder of this report.

II. Summary of the McGill Report

3. The analysis provided by the McGill Report is effectively threefold. First, Dr.

McGill offers affirmative opinions regarding the amount of glutathione (GSH) in the liver and brain, and whether that amount can effectively handle any formation of NAPQI when acetaminophen is used within therapeutic doses. Second, Dr. McGill provides opinions regarding the levels of CYP2E1 expression in the brain and its ability to produce local levels of NAPQI that could lead to toxicity. Third, Dr. McGill opines on the blood-brain barrier and the transport of acetaminophen and its metabolites into the brain. The remainder of this Section will summarize Dr. McGill's affirmative opinions. In the sections that follow, I provide my responses to Dr. McGill's affirmative opinions. I also provide summaries of and responses to Dr. McGill's rebuttal opinions regarding the Louie Report.

4. Dr. McGill claims that the abundance of glutathione in the liver (ranging from 0.5 to 10 mmole/L) and brain (1-2 mmole/L) can effectively handle any formation of NAPQI when acetaminophen is used within therapeutic doses. Dr. McGill's analysis is primarily based on two points. First, with respect to glutathione depletion, Dr. McGill believes that glutathione depletion of greater than 70% is required to induce liver injury, and he concludes that an acetaminophen overdose for an average adult human is approximately 10-15 grams, which is the equivalent to 143-214 mg/kg for a person weighing 70 kg. He references a study from 1974 on acetaminophen-induced liver toxicity to substantiate this claim:

"The normal concentration of glutathione in liver of various animals is about 4 mM [mmol/L]. Liver necrosis occurs in animals after doses of acetaminophen that deplete more than 70% of hepatic glutathione. Assuming a similar glutathione level in man, one would expect that at least 4 mmoles of toxic metabolite is necessary to cause liver injury in man (70% x 1.5 kg liver x 4 mM glutathione). Thus normal individuals would be susceptible to acetaminophen doses over 15 gm (4% of 15 gm

dose = 4 mmoles of metabolite), and patients with induced drug-metabolizing enzymes would be susceptible to doses as low as 10 gm (Fig. 3).’’¹

5. Throughout his report, Dr. McGill’s analysis is fundamentally premised on the assertion that over 70% depletion of glutathione in the liver is necessary for liver injury to occur. As is clear from the analysis, the 70% depletion figure represents the sole basis for Dr. McGill’s claim that “at least 10 g in a single dose is required to cause clinically significant liver injury.” However, the authors of the paper actually stated that “patients with induced drug-metabolizing enzymes would be susceptible doses as low as 10 gm.”² This means that patients who were receiving acetaminophen would need only 66% of the toxic dosage to cause liver injury.

6. Second, Dr. McGill holds that due to the organ-specific nature of drug toxicity, acetaminophen overdose, while potentially damaging to the liver, would likely not injure the brain. He posits that the brain’s glutathione levels are approximately 1-2 $\mu\text{mol/mL}$, a concentration 5 to 10 times lower than the typical hepatic glutathione concentration. This, he indicates, results in a diminished capacity for the brain to neutralize any NAPQI produced within it.

7. Dr. McGill also provides additional opinions regarding the levels of CYP2E1 expression in the brain. Dr. McGill claims that the levels of CYP2E1 expression in the brain are insufficient to produce local levels of NAPQI that could lead to toxicity. In support of this, Dr. McGill calculates that levels of CYP2E1 are 1000-fold lower in the brain than in the liver. Based on his 1000-fold calculation, Dr. McGill postulates, incorrectly, that if any NAPQI is ever generated in the brain after a dose of acetaminophen, it would be immediately neutralized by the ample amount of glutathione present in the brain.

¹ Mitchell JR, Thorgeirsson SS, Potter WZ, Jollow DJ, Keiser H. Acetaminophen-induced hepatic injury: protective role of glutathione in man and rationale for therapy. *Clin Pharmacol Ther.* 1974 Oct;16(4):676-84 (“Mitchell 1974”) (cited in McGill Report, ¶ 30.)

² Mitchell 1974.

8. Finally, Dr. McGill claims that the blood-brain barrier prevents acetaminophen and its metabolites from entering the brain.

9. For the reasons set forth below, Dr. McGill's opinions are flawed and should not be credited.

III. EVALUATION OF THE AFFIRMATIVE OPINIONS IN THE MCGILL REPORT

A. Glutathione Depletion Required for Cellular Injury

10. As stated above, Dr. McGill claims that the abundance of glutathione in the liver (ranging from 0.5 to 10 mmole/L) and brain (1-2 mmole/L) can effectively neutralize any formation of NAPQI when acetaminophen is taken within therapeutic doses. Dr. McGill's analysis hinges on several premises: that over 70% glutathione depletion is required to induce liver injury, that any NAPQI in the brain is immediately detoxified due to glutathione, and that the organ-specific nature of drug toxicity prevents acetaminophen overdose from injuring the brain. However, Dr. McGill's analysis is inaccurate, misinterprets the studies he relies on, and sometimes contradicts his own published research.

11. First, relying on the 70% figure above in justifying his claim that at least 10 grams in a single dose is required for liver injury, Dr. McGill implies throughout his report that similar levels of glutathione depletion are necessary to trigger developmental neurotoxicity through NAPQI expression in the embryonic or fetal brain.

12. Nevertheless, evidence from Dr. McGill's own research suggests otherwise. Dr. McGill's own findings indicate that a greater than 70% reduction is not necessary for NAPQI-induced hepatotoxicity.³ He even cites this study directly within his report. It is unclear to me how

³ McGill MR, Lebofsky M, Norris HR, Slawson MH, Bajt ML, Xie Y, Williams CD, Wilkins DG, Rollins DE, Jaeschke H. Plasma and liver acetaminophen-protein adduct levels in mice after acetaminophen treatment: Dose-Response, mechanisms, and clinical implications. *Toxicology and Applied Pharmacology*. 2013;269(3): 240-9.

Dr. McGill can affirmatively state that a glutathione depletion of greater than 70% is required to induce liver injury while at the same time citing evidence that contradicts this statement. His own research is contradictory, finding that “protein binding can occur without much loss of GSH.”⁴

13. Second, Dr. McGill claims there is immediate detoxification of NAPQI in the brain due to the presence of glutathione. This claim is rooted in his misinterpretation of brain glutathione concentrations. He opines, “Although the typical brain glutathione concentration of 1-2 mmol/L is 5 to 10-fold lower than the typical liver glutathione concentration of 5-10 mmol/L, the levels of CYP2E1 that produce NAPQI are at least 1,000-fold lower in the brain than in the liver.” (McGill Report, ¶ 50.)

14. In his assertions, Dr. McGill opines that the brain is incapable of generating enough NAPQI due to the low level of CYP2E1 found in the brain. He further suggests, “if any NAPQI is ever generated in the brain after a dose of acetaminophen it would be completely detoxified immediately by the substantial amount of glutathione present.” (McGill Report, ¶ 50.) His first assertion that CYP2E1 levels in the brain are 1000-fold lower than in the liver is inaccurate. Dr. McGill stated, “Warner et al. (1988) purified total CYP450 content from rat brain and liver and found that “[t]he yield of P-450 from the whole brain was 90 ± 19 pmol/g of tissue, which is ~1% of the level in liver microsomes. They also measured CYP450 content in various brain regions and found that it never exceeded ~5% of the liver content.” (McGill Report, ¶ 47.) Warner et al. reports that CYP2E1 protein levels ranged from 1-5% when compared to the liver, which is in contrast to Dr. McGill’s assertion.⁵ More importantly, Dr. McGill did not mention that CYP2E1

⁴ *Id.*

⁵ Warner M, Köhler C, Hansson T, Gustafsson JÅ. Regional distribution of cytochrome P-450 in the rat brain: Spectral quantitation and contribution of P-450b, e and P-450c, d. *Journal of Neurochemistry*. 1988;50(4):1057-65. (“Warner 1988”)

is an inducible enzyme where a 10-fold increase has been reported.⁶ In summary, by correcting Dr. McGill's calculations, it becomes clear that NAPQI levels can increase tenfold, due to acetaminophen induction of CYP2E1. This corresponds to up to 50% (5% X 10-fold induction = 50%) of the CYP2E1 activity found in the brain when compared to the liver. However, it is important to bear in mind that the brain's glutathione capacity is only one-fifth as compared to the liver. By using the same calculation (70% X 1.4 kg (brain weight) X 1.5 mM (average glutathione in the brain), it is determined that 5.25 grams of acetaminophen is the threshold leading to neurotoxic effects after a single acetaminophen dose.

15. Third, it is important to note that the effects of prolonged exposure to acetaminophen were not considered in Dr. McGill's analysis. A chronic dose of 1 gm of acetaminophen given every 6 hours for 14 consecutive days led to a 40% increase in acetaminophen levels that corresponded to reduced levels of overall antioxidant capacity which can exacerbate oxidative stress.⁷ Therefore, the long-term effects of acetaminophen and its metabolites on the brain's antioxidant capacity and overall neuronal health cannot be overlooked (as I discuss in more detail below).

16. For all the reasons stated above, it appears that Dr. McGill may have miscalculated the acetaminophen dosage needed to increase fetal/embryo risk for ASD and/or ADHD. In sum, Dr. McGill overlooked that the brain has a lower glutathione capacity than does the liver, he underestimated the level of CYP2E1 in the brain, and he did not take into account the ability to increase expression of CYP2E1 due to acetaminophen induction.

⁶ See Posadas I, Santos P, Blanco A, Muñoz-Fernández M, Ceña V. Acetaminophen induces apoptosis in rat cortical neurons. *PLoS One*. 2010 Dec 10;5(12):e15360. ("Posadas 2010"); Kim SJ, Lee MY, Kwon DY, Kim SY, Kim YC. Alteration in metabolism and toxicity of acetaminophen upon repeated administration in rats. *J Pharmacol Sci*. 2009 Oct;111(2):175-81. ("Kim 2009")

⁷ Nuttall SL, Khan JN, Thorpe GH, Langford N, Kendall MJ. (2003) The impact of therapeutic doses of paracetamol on serum total antioxidant capacity. *J Clin Pharm Ther.*, (4):289-94. ("Nuttall 2003")

B. CYP2E1 Expression

17. Studies cited by Dr. McGill in his report did not specifically analyze CYP2E1 in the brain. Instead, they highlighted the difference in expression of CYP without specifying the isoforms. Contrary to Dr. McGill's assertions, Hansson T et al. showed the brain levels of CYP2E1 using specific antibody staining of brain slices.⁸ In particular, CYP2E1 was expressed in brain glial cells, leading the authors to state, "The present study shows that ethanol-inducible hepatic P450 IIE1 (CYP2E1) is constitutively (without induction) expressed in the rat brain."⁹ They further explained, "The distribution of this P450 (CYP2E1) form in certain populations of nerve cells might have implications for the regioselective toxicity of substrates for P450 IIE1."¹⁰ To illustrate their point, the authors presented micrographs demonstrating the expression of CYP2E1, which are in contrast to Dr. McGill's assertion concerning the low level of expression.¹¹

18. Dr. McGill contends that NAPQI is the cytotoxic metabolite of acetaminophen, however most in vitro and in vivo studies do not use NAPQI; instead, these studies used acetaminophen treatments. Thus, these studies relied on either cellular or animal expression of CYP2E1 to metabolize acetaminophen to form NAPQI. Acetaminophen when co-administered with CYP2E1 inhibitors was found to have reduced acetaminophen-mediated cytotoxic effects.¹² Genetic knock-out of the CYP2E1 (knocking out the expression of CYP2E1) in mice was able to reduce acetaminophen toxicities as well.¹³ These findings suggest acetaminophen treated cells

⁸ Hansson T, Tindberg N, Ingelman-Sundberg M, Kohler C. Regional distribution of ethanol-inducible cytochrome P450 IIE1 in the rat central nervous system. *Neuroscience* 1990;34:451-63.

⁹ *Id.*

¹⁰ *Id.*

¹¹ *Id.*

¹² Kang AM, Padilla-Jones A, Fisher ES, Akakpo JY, Jaeschke H, Rumack BH, Gerkin RD, Curry SC. The Effect of 4-Methylpyrazole on Oxidative Metabolism of Acetaminophen in Human Volunteers. *J Med Toxicol.* 2020 Apr;16(2):169-176.

¹³ Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, Gonzalez FJ. Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem.* 1996 May 17;271(20):12063-7.

required CYP2E1 to catalyze the formation of the toxic acetaminophen metabolite, NAPQI.

19. Posadas et al. evaluated the impact of acetaminophen in cortical neuron cells, and the authors stated that “[i]n vehicle-treated neurons, low levels of CYP2E1 activity were detected (Figure 6A). However, AAP (acetaminophen) treatment for 18h increased CYP2E1 activity in a concentration-dependent manner, indicating that this pathway for the metabolism of AAP (acetaminophen) was increased in cortical neurons.”¹⁴ In addition, the authors showed that acetaminophen can induce an increase in CYP2E1 expression up to 10-fold, which suggests that there is a 10-fold increase in NAPQI formation. Using this data to readjust Dr. McGill’s calculations, the level of NAPQI can be increased by 10 times (due to CYP2E1 induction) or up to 50% (5.0% [upper limits of liver] X 10-fold) of CYP2E1 activity found in the liver. However, the brain glutathione capacity is 5-fold lower than the liver, therefore the levels of brain glutathione may not be able to completely neutralize all of the NAPQI. This further supports the notion that CYP2E1 is an inducible enzyme to levels where adequate NAPQI levels are generated in cortical neuronal cells.

20. Dr. McGill further opines, “Thus, if any NAPQI is ever generated in the brain after a dose of acetaminophen it would be completely detoxified immediately by the substantial amount of glutathione present.” (McGill Report, ¶ 30.) However, not only were Posadas et al. able to determine that CYP2E1 levels were adequate to form NAPQI causing cortical neuronal cell death, but they were also able to recapitulate these cellular findings in animal studies.¹⁵ This prompted the authors to conclude, “The data presented here establish, for the first time, a direct neurotoxic action by AAP (acetaminophen) both in vivo and in vitro in rats at doses below those required to produce hepatotoxicity and suggest that this neurotoxicity might be involved in the general toxic

¹⁴ Posadas 2010.

¹⁵ *Id.*

syndrome...”¹⁶ Posadas et al. emphasized that at below hepatotoxic levels of acetaminophen, the brain levels of acetaminophen and its metabolite, NAPQI, were not adequately neutralized by local glutathione thus allowing cortical neuronal cell death to occur.¹⁷ Posadas et al.’s findings suggest that Dr. McGill’s assertion that the brain levels of CYP2E1 will be inadequate to produce enough NAPQI to be toxic is flawed.

21. In addition, Dr. McGill’s second contention that adequate local glutathione found in the brain can neutralize NAPQI is also flawed. Recent findings report that CYP2E1 expression is not limited to tissues and/or organs but also found ubiquitously in blood, specifically in the plasma in the form of exosomes. Exosomes are small extracellular vesicles produced and extruded into the circulating bloodstream by most cell types. These extracellular vesicles contain bioactive molecules, such as proteins (like CYP2E1), lipids, and nucleic acids (RNA and DNA). The importance of CYP2E1 in the circulating bloodstream was first identified by Kumar et al. who measured the levels of blood carrying exosomes, showing that CYP2E1 was >500 fold higher in than other CYP isoenzymes, and leading the authors to state, “Interestingly, the relative level of CYP2E1 mRNA was >500-fold higher than the other CYPs. The results from the Western blot showed detectable levels of CYP1A1, CYP1B1, CYP2A6, CYP2E1, and CYP3A4.”¹⁸ The authors also stated, “Our results also showed that CYP2E1 is expressed relatively higher in plasma exosomes than hepatic and monocytic cells and exosomes derived from these cells.”¹⁹ These findings suggest that in the presence of acetaminophen, the formation of NAPQI is not limited to organs such as the brain and liver. Rather Kumar et al. suggest that the blood is also a viable

¹⁶ *Id.*

¹⁷ *Id.*

¹⁸ Kumar, S. et al. Specific packaging and circulation of cytochromes P450, especially 2E1 isozyme, in human plasma exosomes and their implications in cellular communications. *Biochemical and biophysical research communications* 491, 675–680.

¹⁹ *Id.*

source of acetaminophen metabolism and conversion to NAPQI.

22. Rahman et al. also reported that CYP2E1 is abundantly present in exosomes derived from healthy human plasma.²⁰ Rahman et al. confirmed Kumar et al.'s discovery of the presence of CYP2E1 in blood-derived exosomes.²¹ The presence of CYP2E1 led Rahman et al. to state, "These exosomes containing increased levels of CYP2E1 caused significant toxicity." Rahman et al. further stated, "we hypothesized that the metabolically active plasma exosomal CYP2E1 cargo participates in cellular pathophysiology and contributes to ALC- (alcohol) and APAP-induced toxicity in hepatic and non-hepatic cells."²² The authors concluded, "Overall, our results showed an important role of exosomal CYP2E1 in exacerbating ALC- (alcohol) and APAP-induced toxicity."²³ Rahman et al.'s finding suggest that acetaminophen can be metabolized in the blood to form NAPQI, which can be transported or diffused into the brain and fetus and cause oxidative stress and apoptosis (cell death).

23. To address whether acetaminophen is present in the fetus/embryo, Rigobello et al. used pregnant rats given subtoxic (35 mg/kg or 350 mg/kg) acetaminophen dosages starting from gestational day 6 until weaning (postnatal day 21).²⁴ Behavioral tests were performed on the offspring at postnatal days 10, 27, and 28. The offspring treated with acetaminophen showed signs of neurological development issues corresponding with increased oxidative stress in brain. The authors found acetaminophen treatment decreased GSH levels in the hippocampus and super oxide dismutase (SOD) activity in the striatum of males exposed to 35 mg/kg of acetaminophen, which

²⁰ Rahman, M.A., Kodidela, S., Sinha, N. et al. Plasma exosomes exacerbate alcohol- and acetaminophen-induced toxicity via CYP2E1 pathway. *Sci Rep* 9, 6571 (2019).

²¹ *Id.*

²² *Id.*

²³ *Id.*

²⁴ Rigobello C, Klein RM, Debiase JD, Ursini LG, Michelin AP, Matsumoto AK, Barbosa DS, Moreira EG. Perinatal exposure to paracetamol: Dose and sex-dependent effects in behaviour and brain's oxidative stress markers in progeny. *Behav Brain Res.* 2021 Jun 25;408:113294. ("Rigobello 2021")

are also subtoxic doses. These results suggest that subtoxic acetaminophen exposure in pregnant rats can decrease brain GSH levels and superoxide dismutase expression in fetal/embryo tissues. These molecular findings showed increases in fetal brain oxidative stress corresponding with poorer neurological development parameters in the offspring. These findings led Rigobella et al. to conclude, “PAR (paracetamol or acetaminophen) treatment decreased hippocampal GSH level and striatal SOD (superoxide dismutase) activity in males exposed to 35 mg/kg, suggesting the vulnerability of these areas in PAR-induced developmental neurotoxicity. Findings suggest PAR (paracetamol or acetaminophen) use during pregnancy and lactation as a potential risk factor for neurodevelopmental disorders with males being more susceptible.”²⁵

24. For all the reasons stated above, Dr. McGill’s opinions concerning CYP2E1 expression are also flawed.

IV. EVALUATION OF THE REBUTTAL OPINIONS IN THE MCGILL REPORT

25. In addition to the affirmative opinions expressed in the McGill Report, Dr. McGill also provides numerous criticisms of the opinions I have expressed in the Louie Report. My responses to these opinions are described below.

A. Allometric Dosing for Animal Models

26. Dr. McGill disagrees with the usage of the allometric scaling method to calculate the dosage of acetaminophen for experimental models. He contends that translating human dosages back to mice is scientifically invalid, even if it follows the FDA's guidance. He asserts, “Plaintiff experts Dr. Cabrera and Dr. Louie try to justify the use of very large doses or concentrations of acetaminophen in experimental models by referencing Human Equivalent Dose (HED) estimates from the U.S. Food and Drug Administration.” (McGill Report, ¶ 20.) He further

²⁵ *Id.*

asserts that “[t]his approach is scientifically invalid.” (McGill Report, ¶ 20.) He contends that it is not correct to translate from humans back to mice, despite the Food and Drug Administration’s (FDA’s) guidance. In particular, he states that a human dosage of 14 mg/kg when translated to mouse dosage using this estimate would surpass the 150 mg/kg dosing threshold leading to hepatotoxicities in mice. However, earlier in his report he stated the following: “Therapeutic use of acetaminophen in humans, which is a dose of about 9 to 14 mg/kg, results in peak concentrations in blood of approximately 66 to 132 $\mu\text{mol/L}$ within about one hour of ingestion.” (McGill Report, ¶ 18.) There he used the higher end of the therapeutic dosage range for children to perform his calculation. However, when these calculations used the lower values in the therapeutic range of 9 mg/kg, the calculated mice dose ($9 \text{ mg/kg} \times 12.3 \text{ (allometric conversion)} = 111 \text{ mg/kg}$) would be 111 mg/kg, which is more than 25% below the hepatotoxic dosage 150 mg/kg threshold for mice.

27. As both Dr. Cabrera and I make clear, it is appropriate to use the allometric scaling method to calculate dosage, which conforms to the official FDA guidance.²⁶ Additionally, the propriety of this approach has been endorsed by numerous other scientists and study authors.

B. Physiologically based Pharmacokinetics of Acetaminophen and Metabolites

28. Dr. McGill criticizes my reference to the paper authored by Mian et al. (2020), which describes a pharmacokinetic model of acetaminophen metabolism in pregnant women. He states in his report, “The model predicted lower maximum and steady-state concentrations of acetaminophen in blood of pregnant women compared to non-pregnant women, indicating that acetaminophen is eliminated from the body faster during pregnancy.” (McGill Report, ¶ 31.) Dr. McGill suggests that Mian et al.’s paper using a physiologically based pharmacokinetic (PBPK)

²⁶ U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). (August 2018). Physiologically Based Pharmacokinetic Analyses—Format and Content, Guidance for Industry. (“FDA Guidance”).

modeling to characterize NAPQI in both pregnant and non-pregnant women was theoretical. He stated, “While the model also predicted greater NAPQI formation in liver in pregnant women, the difference between pregnant and non-pregnant women was marginal.” (McGill Report, ¶ 31.) Dr. McGill trivializes NAPQI elevation in the first trimester of pregnancy (11.03%) when compared to nonpregnant level (7.71%), which he characterized as a “marginal” difference. However, this represents an increase of ~47% as compared to levels found in non-pregnant women. He continues to opine, “Further, because these values for NAPQI formation are derived from a theoretical model, and not from actual observations, their clinical relevance is unknown.” (McGill Report, ¶ 31.) What Dr. McGill is missing is that this predictive modeling was correlated with observed data, which had a strong correlation. The accuracy of drug modelling is accepted by the FDA to evaluate special populations like pregnant females. To this end, the FDA has given guidance about how to conduct these types of analysis. In this regard, the validity of PBPK can be used to support an FDA application.²⁷ Dr. McGill also misunderstands the accuracy of the PBPK modeling methodology where its acceptance by the FDA illustrates its power and accuracy.

C. Levels of Acetaminophen in the Fetal/Embryonic Environment

29. Dr. McGill relies on Wang LH et al. to opine that “[w]hile it is difficult to determine the effect of fetal metabolism in humans empirically due to the highly invasive procedures that would be required, it has been observed that the fetal liver is capable of some degree of acetaminophen metabolism in sheep.” (McGill Report, ¶ 33.) Wang LH et al. concluded that “acetaminophen is transferred across the placenta through passive diffusion into the fetal circulation and is metabolized in the fetus to acetaminophen glucuronide and sulfate.”²⁸ (McGill

²⁷ FDA Guidance.

²⁸ Wang LH, Rudolph AM, Benet LZ. Pharmacokinetic studies of the disposition of acetaminophen in the sheep maternal-placental-fetal unit. *J Pharmacol Exp Ther*. 1986 Jul;238(1):198-205.

Report, ¶ 33.) Wang et al. further validates that acetaminophen can freely cross from maternal to fetal blood, which means that acetaminophen levels in the mother and child can be correlated.²⁹

30. Two studies have shown that CYP2E1 was expressed in fetal brains.³⁰ In Brzezinski et al., the authors concluded, “There was a dramatic increase in human brain CYP2E1 content around gestational day 50 and a fairly constant level was maintained throughout the early fetal period, until at least day 113. The relatively low levels of the P-450 isoform present in conceptual brain may be sufficient to generate reactive intermediates that elicit neuroembryotoxicity ...”³¹ Since acetaminophen can easily traverse the placental barrier and enter into the fetal blood and brain tissue, it is likely that CYP2E1 can metabolize acetaminophen to form NAPQI.

31. These findings are consistent with what Rigobello et al. have shown using subtoxic (35 mg/kg or 350 mg/kg) acetaminophen dosages.³² This study showed offspring from acetaminophen-treated pregnant rats caused neurological development issues in the offspring rats that corresponded with increased oxidative stress in brain. Decreases of glutathione in the hippocampus and super oxide dismutase (SOD) activity in the striatum in the offspring rats are consistent with autism. These molecular findings showed increases in fetal brain oxidative stress that corresponded with poorer neurological development parameters in the offspring. Therefore Dr. McGill’s hypothesis that there is adequate glutathione in the brain to accommodate any NAPQI production is flawed.

²⁹ *Id.*

³⁰ Brzezinski MR, Boutelet-Bochan H, Person RE, Fantel AG, Juchau MR. Catalytic activity and quantitation of cytochrome P-450 2E1 in prenatal human brain. *J Pharmacol Exp Ther*. 1999 Jun;289(3):1648-53 (“Brzezinski 1999”); Boutelet-Bochan H, Huang Y, Juchau MR. Expression of CYP2E1 during embryogenesis and fetogenesis in human cephalic tissues: implications for the fetal alcohol syndrome. *Biochem Biophys Res Commun*. 1997 Sep 18;238(2):443-7.

³¹ Brzezinski 1999.

³² Rigobello 2021.

D. BBB Penetration of Acetaminophen

32. Dr. McGill's report opines the following, "The maximum concentration of acetaminophen measured in CSF did not exceed 52% of the maximum plasma concentration for any of the routes of administration, and in fact was only ~30% for both the oral and i.v. routes." (McGill Report, ¶ 34.) It is important to understand that most drugs have cerebrospinal fluid (CSF) penetration of less than 10%. In this context, a 30% CSF penetration of acetaminophen is considered relatively high in terms of blood-brain barrier (BBB) penetration. Achieving a 30% penetration means that a significant portion of the drug has crossed the BBB capable of entering the CSF and brain tissue. These findings were also confirmed by Posadas et al. who showed high levels of acetaminophen (1 mM acetaminophen in CSF at one hour time point).³³ The pharmacokinetics of acetaminophen in the brain follows the same kinetic features of that of the blood. This suggests that plasma acetaminophen concentration correlates with CSF levels. Posadas et al. correlated the CSF versus plasma levels after a 250 mg/kg intraperitoneal injection of acetaminophen, where the level of CSF versus plasma acetaminophen concentration ratio was close to 100% when using Cmax levels (1 hour after administration), which is substantially higher than 30%.³⁴

33. Moreover, Dr. McGill writes in his report, "One group has claimed that the blood-brain barrier is more permeable to acetaminophen in embryonic rats than adult rats. However, instead of measuring free acetaminophen alone, their assay measured acetaminophen, acetaminophen-glucuronide, acetaminophen-sulfate, and acetaminophen-glutathione all at once. The method they used would detect not only free acetaminophen, but also its harmless acetaminophen-glucuronide, acetaminophen-sulfate, and acetaminophen-glutathione conjugates."

³³ Posadas 2010.

³⁴ *Id.*

(McGill Report, ¶ 34.) In his own words, Dr. McGill states that acetaminophen metabolites reflect that acetaminophen-glutathione was detected. This suggests that acetaminophen not only reaches the fetus, but that the fetus was able to form NAPQI, which is conjugated to form acetaminophen-glutathione. In fact, this data shows that acetaminophen is found in the embryonic/fetal brain, and biotransforming acetaminophen to its metabolite. While Dr. McGill dismisses my opinion that prenatal ingestion of therapeutic doses of acetaminophen produces excess NAPQI levels in the embryonic/fetal brain leading to oxidative stress which increase the risk for development of ASD and ADHD, in fact the papers that he reviewed confirmed that adequate levels of acetaminophen are found in the fetal/embryonic tissue. Moreover, these papers support the ability to metabolize acetaminophen to “acetaminophen-glucuronide, acetaminophen-sulfate, and acetaminophen-glutathione.” (McGill Report, ¶ 34.) While these conjugates of acetaminophen are not toxic, the presence of APAP-GSH suggests that NAPQI is formed in the fetus. This further supports the notion that NAPQI-protein adducts, which are surrogate markers of cellular toxicities, will also be present.

34. In this context, Dr. McGill’s argument that BBB prevents acetaminophen from entering the CSF and brain tissue is invalid, because even 30% penetration in the CSF suggests high levels of acetaminophen.³⁵ Moreover, his references further underscore the point that the fetus/embryo is able to metabolize acetaminophen to form the toxic NAPQI. This is affirmed by the presence of acetaminophen-glutathione which is also known as NAPQI-glutathione. Taken together, the foregoing demonstrates that 1) the concentration of acetaminophen in the fetus/embryo is high, 2) the fetus/embryo is able to metabolize acetaminophen to form the NAPQI,

³⁵ Singla NK, Parulan C, Samson R, Hutchinson J, Bushnell R, Beja EG, et al. Plasma and cerebrospinal fluid pharmacokinetic parameters after single-dose administration of intravenous, oral, or rectal acetaminophen. *Pain Pract.* 2012;12:523–532. (“Singla 2012”)

and 3) NAPQI can bind to glutathione in neurons, thus depleting glutathione, increasing oxidative stress, and leading to apoptosis.

E. Conclusion

35. In his report, Dr. McGill further asserts, “In the studies where the enzymatic activity of CYP2E1 was measured in brain, it was extremely low. Because CYP2E1 is the primary enzyme responsible for producing NAPQI, it is not biologically plausible that excess levels of NAPQI would be produced in human embryonic/fetal brain after therapeutic doses of acetaminophen are ingested by pregnant women.” (McGill Report, ¶ 48.)

36. Dr. McGill’s conclusion is flawed for the following reasons. First, he asserts that the enzymatic activity of CYP2E1 in the brain is “extremely low,” but falsely represents that the levels of CYP2E1 are much lower than what Warren et al.’s research actually finds. (McGill Report, ¶ 48.) Dr. McGill claims that levels of CYP2E1 in the brain are 1,000-fold lower than levels in the liver, but when Warren et al. measured the protein levels of CYP2E1, they found levels between 1-5% of that found in the liver.³⁶

37. Second, Dr. McGill neglected to consider that CYP2E1 is not only in the brain, but its expression is inducible. This means that the presence of acetaminophen can cause the brain to make more CYP2E1. This was shown by Kim et al. and Posadas et al., who both demonstrated that acetaminophen can increase CYP2E1 by 10-fold.³⁷ Interestingly, Mitchell et al. also recognized the impact of cytochrome p450 system (e.g., CYP2E1) induction, where he stated, “patients with induced drug-metabolizing enzymes would be susceptible (to hepatic toxicity) doses as low as 10 gm.”³⁸ This means that patients who were receiving acetaminophen would need only

³⁶ Warner 1988.

³⁷ Kim 2009; Posadas 2010.

³⁸ Mitchell 1974.

66% of the toxic dosage to cause liver injury, which represented a 33% reduction of acetaminophen necessary to cause liver injury.

38. The inducible nature of CYP2E1 suggests that chronic acetaminophen can provide a consistent level of NAPQI that be produced by the liver and exosomal CYP2E1 found in the blood. In women chronically taking acetaminophen for 28 days or more, it is conceivable that they will generate more CYP2E1 and thus form 10-fold more NAPQI, which can cause tissue damage such as fetal/embryo brain tissue. In addition, chronic use of acetaminophen can reduce overall antioxidant capacity and thus reduce the ability to neutralize NAPQI.

39. Third, Dr. McGill incorrectly assumes that acetaminophen has poor brain penetration. This assumption is also flawed, where a range of 30% to 52% of the acetaminophen in the blood is found in the CSF, a surrogate marker of the brain tissue concentration.³⁹ Together, the data show that acetaminophen can freely penetrate the brain tissue where, in the presence of increased CYP2E1 expression, it will lead to significantly higher NAPQI in brain tissue.

40. Dr. McGill concluded that “it is not biologically plausible that excess levels of NAPQI would be produced in human embryonic/fetal brain after therapeutic doses of acetaminophen are ingested by pregnant women.” (McGill Report, ¶ 48.) By contrast, Rigobello et al. showed that when using subtoxic acetaminophen levels, fetal/embryo showed signs of neurological development issues corresponding with increases in brain oxidative stress.⁴⁰ These results suggest that acetaminophen exposure of fetal/embryo at these subtoxic dosages can cause neurological development issues which are correlated with behavioral changes. These findings raise concerns about the potential risks of acetaminophen use during pregnancy and lactation as a

³⁹ Singla 2012.

⁴⁰ Rigobello 2021.

contributing factor to neurodevelopmental disorders.⁴¹

V. RESPONSE TO THE POWELL REPORT

41. As mentioned above, Defendants' expert Dr. Powell also criticizes portions of my opinions for various reasons. Although I do not respond to each one of Dr. Powell's criticisms, that is not an indication that I agree with or concede to any of his points. To the contrary, and as explained below, Dr. Powell's views as they concern my opinions are oftentimes mistaken or inaccurate.

A. Reliance on Gervin et al.

42. Specifically, Dr. Powell disagrees with my reliance on the Gervin et al. study,⁴² the results of which he says were not replicated in the Olstad et al. (2023) study.⁴³ (Powell Report, ¶ 227.) But Dr. Powell's criticisms are misguided, for a few reasons. First, Olstad conceded that different studies have found different DNA methylation levels in relation to maternal folic acid intake during pregnancy. Multiple studies have demonstrated that folic acid supplementation can cause changes in DNA methylation and mitigate the toxic effects of acetaminophen.⁴⁴

43. Second, the Olstad et al. study's negative findings do not diminish the positive

⁴¹ *Id.*

⁴² Gervin K, Nordeng H, Ystrom E, Reichborn-Kjennerud T, Lyle R. Long-term prenatal exposure to paracetamol is associated with DNA methylation differences in children diagnosed with ADHD. *Clin Epigenetics*. 2017 Aug 2;9:77.

⁴³ Olstad EW, Nordeng HME, Lyle R, Gervin K. No impact of prenatal paracetamol and folic acid exposure on cord blood DNA methylation in children with attention-deficit/hyperactivity disorder. *Front Genet*. 2023 Jun 15;14:1204879.

⁴⁴ See, e.g., Joubert, B. R., Den Dekker, H. T., Felix, J. F., Bohlin, J., Ligthart, S., Beckett, E., et al. (2016). Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. *Nat. Commun.* 7, 10577; Ondičová, M., Irwin, R.E., Thursby, S.J. et al. Folic acid intervention during pregnancy alters DNA methylation, affecting neural target genes through two distinct mechanisms. *Clin Epigenet* 14, 63 (2022); Lee SJ, Kang MH, Min H. Folic acid supplementation reduces oxidative stress and hepatic toxicity in rats treated chronically with ethanol. *Nutr Res Pract*. 2011 Dec;5(6):520-6; Asbaghi O, Ghanavati M, Ashtary-Larky D, Bagheri R, Rezaei Kelishadi M, Nazarian B, Nordvall M, Wong A, Dutheil F, Suzuki K, Alavi Naeini A. Effects of Folic Acid Supplementation on Oxidative Stress Markers: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Antioxidants (Basel)*. 2021 May 28;10(6):871.

findings by Addo⁴⁵ and Eslamimehr,⁴⁶ both of which analyzed different cohorts and were consistent with the findings of Gervin et al. It is possible that differences in methodology, bucketing of data, and the study's analysis plan can explain the different results between Olstad and Gervin.

44. Third, the Olstad et al. study employed a different technology for detecting DNA methylation differences than the one used by Gervin et al. Olstad analyzed approximately 850,000 CpG sites across the genome, whereas Gervin analyzed approximately 450,000 CpG sites at CpG islands and promoters. This is also a potential methodological difference that may explain the differences in the outcomes of the Olstad and Gervin studies.

B. Reliance on Jetten et al.

45. Dr. Powell also takes issue with my reliance on the Jetten et al. study. Dr. Powell asserts that the Jetten et al. study only involved 7 subjects. (Powell Report, ¶ 189.) While this appears to be a small universe of participants, Dr. Powell neglects to mention that the study design was the most rigorous pharmacological study design. In this design, all of the participants were assessed at three different acetaminophen dosages, meaning there were actually 21 participants across all of the dosage groups.⁴⁷ More importantly, this design reduces the issue of intersubject variability.

46. Jetten et al. used the crossover design to compare the effects of dosages of

⁴⁵ Addo KA, Bulka C, Dhingra R, Santos HP Jr, Smeester L, O'Shea TM, Fry RC. Acetaminophen use during pregnancy and DNA methylation in the placenta of the extremely low gestational age newborn (ELGAN) cohort. *Environ Epigenet.* 2019 Aug 6;5(2):dvz010.

⁴⁶ Eslamimehr S, Jones AD, Anthony TM, Arshad SH, Holloway JW, Ewart S, Luo R, Mukherjee N, Kheirkhah Rahimabad P, Chen S, Karmaus W. Association of prenatal acetaminophen use and acetaminophen metabolites with DNA methylation of newborns: analysis of two consecutive generations of the Isle of Wight birth cohort. *Environ Epigenet.* 2022 Feb 2;8(1):dvac002.

⁴⁷ Jetten MJA, Gaj S, Ruiz-Aracama A, de Kok TM, van Delft JH, Lommen A, van Someren EP, Jennen DG, Claessen SM, Peijnenburg AA, Stierum RH, Kleijnans JC (2012). 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. *Toxicology and applied pharmacology*, 259(3), 320–328. ("Jetten 2012")

acetaminophen within the same group of participants.⁴⁸ By using the same subjects for each intervention, researchers can reduce individual variability and increase the statistical power. This vigorous study design ensured that there were no residual effects where participants had a wash out period between each phase.

47. While Dr. Powell criticizes the number of participants, he did not argue the validity of the finding. This is predicated on not only the study design which reduced variability, but also the comprehensive nature of metabolomics (comprehensive evaluation of acetaminophen and its metabolites) combined with transcriptomics (gene expression changes in relation to the blood levels of acetaminophen and its metabolites). The findings from this study suggest that there was subclinical or occult liver injury occurring even at therapeutic dosages of acetaminophen.⁴⁹ Unfortunately, Dr. Powell's objections did not take into account the vigorous nature of the study design, which mitigates the most problematic factor, intersubject variability.

C. Reliance on Nuttall et al.

48. Dr. Powell in his report criticizes my citation of Nuttall's 2003 study, suggesting that it offers no explanation of how a 10% decline in antioxidant capacity in healthy, non-pregnant volunteers would be relevant to pregnant women or developing fetuses. (Powell Report, ¶ 190.) However, Dr. Powell overlooks the fundamental deduction that if healthy young volunteers experience a 10% reduction of total antioxidant capacity (TAC) after 14 days of continuous acetaminophen exposure, pregnant women taking the same therapeutic dose of acetaminophen should expect a similar reduction in TAC. Nuttall's study only evaluated the effects of a 14-day treatment period.⁵⁰ Hence, it is expected that pregnant mothers taking more than 28 days of

⁴⁸ *Id.*

⁴⁹ *Id.*

⁵⁰ Nuttall 2003.

acetaminophen will have similar, if not greater, reduction in total antioxidant capacity. This provides important context when combined with Jetten et al.'s data, which showed participants taking 1 gm acetaminophen 4 times a day developing biomarkers consistent with hepatotoxicity.⁵¹ Taken together, if subclinical hepatotoxicity is occurring in the liver that has a large glutathione store, what impact will this have on the fetus/embryo in mothers who are taking therapeutic dosages of acetaminophen. While Dr. Powell may not be aware that acetaminophen can freely traverse the placental barrier, this is noteworthy because it signifies fetal CYP2E1 can form the necessary NAPQI to cause tissue injury.

D. Reliance on Posadas et al.

49. Dr. Powell criticizes my reliance on Posadas 2010, arguing that the study's use of high acetaminophen concentrations makes it far removed from the real-life scenario of a human fetus exposed to acetaminophen *in utero*. (Powell Report, ¶ 213.) However, Posadas 2010 analyzed therapeutic levels of acetaminophen below hepatotoxic levels.⁵² Moreover, other studies corroborate Posadas 2010. For example, Heard 2011 demonstrated that acetaminophen-cysteine levels, a serum biomarker of acetaminophen exposure, in people taking therapeutic doses of acetaminophen can be of the same order of magnitude as the levels found in people taking supratherapeutic (toxic) doses of acetaminophen.⁵³ In other words, lower, therapeutic doses of acetaminophen can elicit a similar toxic effect as higher, supratherapeutic doses of acetaminophen.

E. Reliance on Anand et al.

50. Dr. Powell stated that the findings are reciprocal to what the authors originally

⁵¹ Jetten 2012.

⁵² Posadas 2010.

⁵³ Heard KJ, Green JL, James LP, Judge BS, Zolot L, Rhyee S, Dart RC. Acetaminophen-cysteine adducts during therapeutic dosing and following overdose. *BMC Gastroenterol*. 2011 Mar 14;11:20. doi: 10.1186/1471-230X-11-20.

hypothesized that decreased GSH would correlate with an increase in amino acid. (Powell Report, ¶ 198.) While the authors' transparency is refreshing, their finding may be consistent with an increase in 8-OHdG (8-hydroxy-2'-deoxyguanosine) which correlated with acetaminophen concentration in the cord. Increased oxidative stress should degrade proteins and increase free levels of amino acid.

51. What is important from Anand et al.⁵⁴ is that there is a correlation between acetaminophen and 8-OHdG (8-hydroxy-2'-deoxyguanosine). There is a direct correlation between oxidative stress with acetaminophen. The findings from Anand et al. are consistent with those published by Powell et al.⁵⁵ In their findings, Powell et al. showed, "Oxidative stress in liver was evaluated by a diverse panel of markers that included assessing expression of base excision repair (BER) genes, quantifying oxidative lesions in genomic DNA, and evaluating protein and lipid oxidation. A subtoxic dose of APAP produced significant accumulation of nitrotyrosine protein adducts. Both subtoxic and toxic doses caused a significant increase in 8-hydroxy-deoxyguanosine (8-OH-dG) as well as a significant decrease in glutathione (GSH) content."⁵⁶

VI. CONCLUSION

52. As set forth in the preceding sections of this report, I am able to draw the preceding conclusions with a reasonable degree of scientific certainty.

⁵⁴ Anand NS, Raghavan R, Wang G, Hong X, Azuine RE, Pearson C, Zuckerman B, Xie H, Wang X. Perinatal Acetaminophen Exposure and Childhood Attention-Deficit/Hyperactivity Disorder (ADHD): Exploring the Role of Umbilical Cord Plasma Metabolites in Oxidative Stress Pathways. *Brain Sci.* 2021 Sep 30;11(10):1302.

⁵⁵ Powell CL, Kosyk O, Ross PK, Schoonhoven R, Boysen G, Swenberg JA, Heinloth AN, Boorman GA, Cunningham ML, Paules RS, Rusyn I. Phenotypic anchoring of acetaminophen-induced oxidative stress with gene expression profiles in rat liver. *Toxicol Sci.* 2006 Sep;93(1):213-22.

⁵⁶ *Id.*

All opinions offered herein are held to a reasonable degree of scientific certainty.

Dated: July 28, 2023

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Stan L", with a long horizontal flourish extending to the right.

Stan G. Louie, Pharm.D.